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A Truncated Diphtheria Toxin Based Recombinant Porcine CTLA-4 Fusion Toxin

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Abstract

Targeted cell therapies are possible through the generation of recombinant fusion proteins that combine a toxin, such as diphtheria toxin (DT), with an antibody or other molecule that confers specificity. Upon binding of the fusion protein to the cell of interest, the diphtheria toxin is internalized which results in protein synthesis inhibition and subsequent cell death. We have recently expressed and purified the recombinant soluble porcine CTLA-4 both with and without *N*-glycosylation in yeast *Pichia Pastoris* for *in vivo* use in our preclinical swine model. The glycosylated and non-*N*-glycosylated versions of this recombinant protein each bind to a porcine CD80 expressing B-cell lymphoma line (LCL13271) with equal affinity ($K_D = 13$ nM). In this study we have linked each of the glycosylated and non-*N*-glycosylated soluble porcine CTLA-4 proteins to the truncated diphtheria toxin DT390 through genetic engineering yielding three versions of the porcine CTLA-4 fusion toxins: 1) monovalent glycosylated soluble porcine CTLA-4 fusion toxin; 2) monovalent non-*N*-glycosylated soluble porcine CTLA-4 fusion toxin and 3) bivalent non-*N*-glycosylated soluble porcine CTLA-4 fusion toxin. Protein synthesis inhibition analysis demonstrated that while all three fusion toxins are capable of inhibiting protein synthesis *in vitro*, the non-*N*-glycosylated porcine CTLA-4 isoforms function most efficiently. Binding analysis using flow cytometry of the porcine CTLA-4 fusion toxins to LCL13271 cells also demonstrated that the non-*N*-glycosylated porcine CTLA-4 isoforms bind to these cells with higher affinity compared to the glycosylated fusion toxin. The monovalent non-*N*-glycosylated porcine CTLA-4 fusion toxin was tested *in vivo*. *NSG* (NOD/SCID IL-2 receptor $\gamma^{-/-}$) mice were injected with porcine CD80⁺ LCL13271 tumor cells. All animals succumbed to tumors and those treated with the monovalent non-*N*-glycosylated porcine CTLA-4 fusion toxin survived longer based on a symptomatic scoring system compared to the untreated controls. This recombinant

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protein may therefore provide a novel approach for *in vivo* depletion of porcine antigen presenting cells (APCs) for studies investigating the induction of transplantation tolerance, autoimmune disease and cancer treatment.

Keywords

Fusion toxin; Diphtheria toxin; porcine CTLA-4; porcine antigen presenting cell; *Pichia pastoris* expression

1. Introduction

Blocking co-stimulation pathways has been broadly utilized to modulate the immune response for studies involving transplantation tolerance and autoimmunity. Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4), also known as CD152, is an important co-stimulatory molecule which serves as a negative regulator for T cell proliferation and differentiation. The CTLA-4/CD28-CD80/CD86 pathway is a critical co-stimulatory pathway for adaptive immune responses. T cell activation leads to increased expression of CTLA-4, an inhibitory receptor which binds to CD80 and CD86 on APCs. CTLA-4 depletes CD80 and CD86 ligands of neighboring APCs through trans-endocytosis to impair the CD28-CD80/CD86 stimulation so as to down regulate the T-cell immune response (Qureshi et al., 2011).

In our lab we have a unique large animal model, the MGH MHC-defined miniature swine, for studying immune regulation and transplantation tolerance (Sachs et al., 1976; Sachs, 1992). We have successfully expressed and purified glycosylated and non-*N*-glycosylated soluble porcine CTLA-4 proteins in yeast *Pichia pastoris* (Peraino et al., 2012). Both glycosylated and non-*N*-glycosylated soluble porcine CTLA-4 each bind to a porcine CD80-expressing B-cell lymphoma line (LCL13271) ($K_D = 13$ nM) and competitively inhibit the binding of an anti-CD80 monoclonal antibody.

The truncated diphtheria toxin DT390, which contains only the catalytic domain (A chain) and translocation domain (B chain) has been successfully used to build recombinant immunotoxins (Woo et al., 2002; Kim et al., 2007; Wang et al., 2011). In this study we took advantage of the high-binding affinity interaction between CTLA-4 and CD80/CD86 and genetically fused each of the glycosylated and non-*N*-glycosylated soluble porcine CTLA-4 proteins to DT390 yielding porcine CTLA-4 fusion toxins. We confirmed the functional ability of these reagents using an *in vitro* protein synthesis inhibition assay. Binding specificity and affinity to the target cells was analyzed using flow cytometry. Target cell depletion *in vivo* was determined using a newly developed porcine B-cell lymphoma tumor mouse model in which *NSG* mice were injected with LCL13271 cells. The efficacy of the non-*N*-glycosylated fusion toxin was then analyzed by observing tumor progression in these mice compared to untreated controls.

2. Materials and methods

2.1. Plasmid Construction

The codon-optimized DT390 nucleotide sequence (Woo et al., 2002) was used for the DT390 domain and has been modified to include an N-terminal alanine (A) and double mutations (dm) to prevent glycosylation in the eukaryotic expression system *Pichia pastoris* (Woo et al., 2002; Liu et al., 2000). Codon-optimized glycosylated and non-*N*-glycosylated soluble porcine CTLA-4 nucleotide sequences (Peraino et al., 2012) were applied to generate the porcine CTLA-4 domains. A strategy similar to that used to construct A-dm-DT390biscFv (2-6-15) in *Pichia pastoris* (Wang et al., 2011) was employed to build these

fusion toxins. As shown in Figure 1, the bisCFv (2-6-15) was replaced using the codon-optimized glycosylated or non-*N*-glycosylated soluble porcine CTLA-4. The non-*N*-glycosylated bi-porcine CTLA-4 version was formed by linking two codon-optimized non-*N*-glycosylated soluble porcine CTLA-4 proteins using a (G₄S)₃ linker consisting of three tandem chains each made of four glycines and a serine residue. To facilitate later purification six histidines (6x His tag) were added to the C-terminus of each fusion protein. To construct DT390-pCTLA-4-Gly or DT390-pCTLA-4-Non-*N*-Gly, the codon-optimized glycosylated or non-*N*-glycosylated soluble porcine CTLA-4 DNA (Peraino et al., 2012) was amplified using PCR primers DTCTL carrying *Nco*I site and CTL161 carrying an *Eco*RI site then cloned into pwPICZalpha-DT390 (Wang et al., 2011) between *Nco*I and *Eco*RI sites yielding the final construct DT390-pCTLA-4-Gly or DT390-pCTLA-4-Non-*N*-Gly in pwPICZalpha. To construct DT390-bi-pCTLA-4-Non-*N*-Gly, the first soluble porcine CTLA-4-Non-*N*-Gly was amplified using PCR primers DTCTL carrying *Xho*I and *Nco*I sites + CTL-E carrying *Bam*HI and *Eco*RI sites then cloned into pwPICZalpha between *Xho*I and *Eco*RI sites for sequencing confirmation. The insert was subsequently cut out with *Nco*I + *Bam*HI as insert I. The second soluble porcine CTLA-4-Non-*N*-Gly was PCR amplified using CTL-B carrying *Xho*I and *Bam*HI sites + CTL161 carrying an *Eco*RI site then cloned into pwPICZalpha between *Xho*I and *Eco*RI sites for sequencing confirmation. The insert was then cut out with *Bam*HI + *Eco*RI as insert II. The insert I carrying *Nco*I and *Bam*HI sites + insert II carrying *Bam*HI and *Eco*RI sites (*Nco*I-pCTLA-4-Non-*N*-Gly-*Bam*HI/*Bam*HI-pCTLA-4-Non-*N*-Gly-*Eco*RI) were together cloned into pwPICZalpha-DT390 between *Nco*I and *Eco*RI yielding the final construct DT390-bi-pCTLA-4-Non-*N*-Gly in pwPICZalpha. DT390 was amplified using PCR primers DT390 For carrying *Xho*I site + DT390 Rev carrying *Eco*RI site and then cloned into pwPICZalpha (Peraino et al., 2012) between *Xho*I and *Eco*RI sites yielding DT390 in pwPICZalpha. All PCR primers that were used are listed in Table 1.

2.2. Protein Expression in *Pichia pastoris*

Approximately 5–10 µg of the above constructed plasmid DNA was linearized by *Sac*I digestion for 3 h at 37°C, treated with Qiagen PCR purification kit then transformed into a diphtheria-toxin resistant yeast *Pichia pastoris* strain mutEF2JC307-8(2) (Liu et al., 2003). The transformants were selected on YPD plates containing Zeocin (100 µg/ml). The Excella E24 incubator shaker (New Brunswick) was employed to express the porcine CTLA-4 fusion toxins. Six colonies were randomly picked and cultivated in small tubes containing 5 mL YPD (1% yeast extract, 2% peptone and 2% dextrose) at 30°C with shaking at 250 rpm for 24h as growth phase I. Cultures were then centrifuged and cell pellets were re-suspended in 5 mL of YPG (1% yeast extract, 2% peptone, 1% glycerol) at 30°C with shaking at 250 rpm for another 24h as growth phase II. The cultures were induced with methanol in 2 mL BMMYC (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 7.0, 1.34% yeast nitrogen base without amino acids, 4×10^{-5} % biotin, 0.5% methanol and 1% casamino acids) for 48h at 25°C with shaking at 225 rpm. Antifoam (Emerald Performance Materials, Cat# KFO673) was added at a concentration of 0.02% (v/v) to all of the growth and induction media. Phenylmethanesulfonyl fluoride (PMSF) (Sigma) was added at a final concentration of 1 mM to the methanol solution to inhibit protein degradation during the induction phase. The culture supernatants were analyzed by SDS-PAGE under non-reducing conditions.

One clone was selected from the above small scale analysis for large-scale expression which was then performed using the same procedure described above scaled up. The seed culture was prepared by inoculating a single colony into YPD media, then incubating at 25°C, 225 rpm over the weekend (~64 h). Antifoam, PMSF and penicillin/streptomycin were added to the media at the same concentrations as mentioned previously for the small-scale expression.

Five percent (v/v) of this seed culture was transferred to 1L PYREX shake flasks containing 250 mL YPD media and cultured at 30°C, 250 rpm for 24 hours. Subsequently, cells were centrifuged at 1500 rpm for 5 minutes and the cell pellet was re-suspended in 250 mL YPG media then cultured at 30°C, 250 rpm for 24 hours. For induction cells were centrifuged at 1500 rpm for 5 minutes and the cell pellet was re-suspended in 125 mL BMMYC induction media and induced at 25°C, 225 rpm for 48 hours. Following induction, 0.5% methanol was added twice daily (at 5 h, 24 h, and 30 h after initial induction) to sustain the methanol levels. After 48 h induction, yeast cells were pelleted by centrifugation at 3000 rpm, 4°C for 10 minutes. Thereafter, the supernatant, containing the porcine CTLA-4 fusion toxin was purified.

2.3. Protein Purification

Ni-Sepharose[™] 6 fast flow resin was packed in a 5 cm x 20 cm XK50 column (GE healthcare Cat#18-1000-71) for the first purification. The column was equilibrated with 10 column volumes (CVs) of 20 mM sodium phosphate pH 7.4, 0.5 M NaCl, and 5 mM imidazole. The sample was prepared by adding 0.5 M NaCl, 20 mM sodium phosphate pH 7.4, and 5 mM imidazole, then filtered through crepe fluted filter paper (VWR) and loaded onto the equilibrated column. The column was washed using 6 CVs of 20 mM sodium phosphate pH 7.4, 0.5 M NaCl, and 5 mM imidazole. The bound proteins were eluted with 20 mM sodium phosphate pH 7.4, 0.5 M NaCl, 500 mM imidazole into eight 1 CV fractions. The purification fractions were analyzed using 4–12% NuPAGE® Bis-Tris gel and stained with GelCode Blue stain reagent (Thermo Scientific). The fractions containing the protein of interest were pooled then dialyzed using a 3.5 kDa cut off Spectra/Por® membrane tubing (Spectrumlabs) against 4L of 20 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 5% glycerol at 4°C overnight with constant stirring and one replacement of dialysis buffer.

Strong anion exchange resin Poros® 50 HQ (Applied Biosystems) in a XK16/20 column (GE Healthcare) was employed for the second purification. The column was equilibrated with 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 5% glycerol (10 CVs). The dialyzed sample was loaded onto the column and washed with 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 5% glycerol (8 CVs). The bound protein was eluted first with eight 1 CV fractions of 200 mM sodium borate in 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 5% glycerol then eight 1 CV fractions of 200 mM sodium borate + 50 mM NaCl in 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 5% glycerol. The purified fractions were analyzed using 4–12% NuPAGE Bis-Tris SDS gels. Fractions containing the protein of interest were pooled and dialyzed as described above against 4 L of 1xPBS + 5% glycerol. Protein concentration was determined using Pierce BCA protein assay kit (Thermo Scientific). The purification for DT390 alone was performed as previously described (Wang et al., 2011).

2.4. Western Blot Analysis

Protein samples were separated by electrophoresis using a NuPAGE 4–12% Bis-Tris Gel. The proteins were then electro-transferred at 35 V for 1 h onto a nitrocellulose membrane filter paper (Invitrogen) using 1x NuPAGE; transfer buffer (Invitrogen). Thereafter, the membrane was blocked with 5% non-fat dry milk (Bio-Rad) in 1xPBS, pH 7.4, 0.02% Tween 20 for 1 h with shaking at room temperature. Subsequently the non-fat dry milk was poured off and the membrane was washed once for 5 minutes with 1xPBS, pH 7.4, 0.02% Tween 20 at room temperature with shaking. Mouse anti-His monoclonal antibody (1.11 µg/mL, 4A12E4, Invitrogen) or mouse anti-diphtheria toxin monoclonal antibody (8.5 µg/mL, clone# 3B6, cat# C86036M, Meridan) in 5% non-fat dry milk in 1xPBS, pH 7.4, 0.02% Tween 20 was added to the membrane and allowed to shake for 1 h at room temperature. The membrane was then washed for three times as described above. Rat anti-mouse IgG-HRP (1.25 µg/mL, Invitrogen) in 5% non-fat dry milk in 1xPBS, pH 7.4, 0.02% Tween 20

was added to the membrane and incubated at room temperature for 1 h with shaking to detect the bound antibody-fusion toxin complexes. The membrane was then washed for three times as described above. Protein detection was achieved using TMB membrane peroxidase substrate (KPL Cat#:50-77-02). Color development was stopped with dH₂O and the membrane was allowed to dry overnight at room temperature in the dark.

2.5. Biotin-labeling of the Porcine CTLA-4 Fusion Toxins

Each of the porcine CTLA-4 fusion toxins was labeled with EZ-Link Sulfo-NHS Biotin (Pierce, Cat# 21217). One milligram of NHS-Biotin was added to one milligram of porcine CTLA-4 fusion toxin then the reaction was incubated for 2 hours at 4°C with rocking. The reaction was transferred to a Slide-a-lyzer dialysis cassette, 10K MWCO, 0.5–3 mL (Thermo Fisher Cat# 66380) and dialyzed against 2 L of 1xPBS for 24 hours at 4°C with constant stirring, changed once.

2.6. Flow Cytometry Binding Analysis of Porcine CTLA-4 Fusion Toxins to Porcine CD80

LCL13271(Cho et al., 2007) cells were cultured as previously described (Peraino et al., 2012). Cells were stained with porcine CTLA-4 fusion toxin at a range of dilutions. Biotin-labeled hamster anti-mouse CD80 mAb clone 16-10A1 (BioLegend, Cat# 104703) was used as a positive control at a final concentration of 17 nM. Negative control cells were stained with only streptavidin-PE at a final concentration of 1.5 ng/uL (without biotylated porcine CTLA-4 fusion toxin). Flow cytometry was carried out using a Becton Dickson FACScan and analysis was performed using Winlist analysis software (Verity Software House, Topsham, ME).

2.7. K_D Determination

Flow cytometry was used to analyze the binding affinity of the porcine CTLA-4 fusion toxins. The dissociation constant for each fusion toxin was determined and binding affinity was inferred based on the calculated K_D values. These assays were performed using LCL13271 cells with a wide range of concentrations of each of the biotinylated porcine CTLA-4 fusion toxins. The mean fluorescence intensity (MFI) values were tabulated and plotted on a linear scale versus each biotinylated porcine CTLA-4 fusion toxin concentration. This plot was fitted using non-linear least-squares by KaleidaGraph software (Synergy Software, Reading PA). The dissociation constant (K_D) was determined using the hyperbolic equation $y = m1 + m2 * m0 / (m3 + m0)$, where y = MFI at the given biotinylated porcine CTLA-4 fusion toxin concentration, $m0$ = biotinylated porcine CTLA-4 fusion toxin concentration, $m1$ = MFI of zero biotinylated porcine CTLA-4 fusion toxin control, $m2$ = MFI at saturation and $m3 = K_D$, the equilibrium dissociation constant (Yeast Display scFv Antibody Library User's Manual, Pacific Northwest National Laboratory, Richland, WA; <http://www.sysbio.org/dataresources/index.stm>).

2.8. Binding Specificity of the Porcine CTLA-4 Fusion Toxins to Porcine CD80

LCL13271 cells were cultured as previously described (Peraino et al., 2012). The cells were re-suspended to a concentration of 1.0×10^7 cells/mL in FACS media [1x Hanks Balanced Salt Solution (HBSS) with Ca⁺ and Mg⁺, (Invitrogen Cat. #14065-056), 0.1% Bovine Serum Albumin (BSA) (Fraction V VWR Cat. #EM-2930), 0.1% Sodium Azide (Sigma, Cat. #S2002), 0.03% Sodium Bicarbonate (Fisher, Cat. #BW15-6131)] and aliquotted into FACS tubes at 1.0×10^6 cells / tube. Unlabeled porcine CTLA-4 fusion toxin was then added to LCL13271 cells at a range of concentrations and allowed to incubate for 5 minutes at 4°C in the dark. After five minutes, biotin-labeled hamster anti-mouse CD80 mAb clone 16-10A1 (BioLegend, Cat#104703) at a final concentration of 17 nM or biotinylated soluble porcine CTLA-4 was added to each tube and the cell suspensions were incubated for 30 minutes at

4°C in the dark. Cells were washed twice with 2 mL of FACS media and centrifuged at 1200 rpm, 4°C for 5 minutes. Streptavidin-PE was added to each tube at a final concentration of 1.5 ng/uL and the tubes were then incubated for 15 minutes at 4°C in the dark. Cells were subsequently washed once as described above then re-suspended in 500 µL of FACS media. Flow cytometry was carried out using a Becton Dickson FACScan and data were analyzed using Winlist analysis software (Verity Software House, Topsham, ME).

2.9. Protein Synthesis Inhibition

Porcine CTLA-4 fusion toxins (DT390-pCTLA-4-Gly, DT390-pCTLA-4-Non-*N*-Gly, and DT390-bi-pCTLA-4-Non-*N*-Gly) were diluted to 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} and 10^{-10} M in 1x leucine-free RPMI 1640 media supplemented with 12% fetal bovine serum, 10 mM hepes (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid), 1x nonessential amino acids, 1mM sodium pyruvate, 2mM glutamine, and 2.5×10^{-5} M 2-mercaptoethanol. Ten microliters of fusion toxin dilutions were added to each well (three wells per dilution) in a 96-well flat bottom plate (Corning). LCL13271 cells were washed twice by centrifugation at 1000 rpm, 20°C for 5 minutes in 50 mL of leucine-free RPMI 1640 media described above. Cells were then diluted to 5.0×10^5 cells/mL in leucine-free RPMI 1640 media and 100 µL of cells was added to each well containing the fusion toxin to achieve a final cell concentration of 5.0×10^4 cells/well. Six wells contained only cells, three of these remained without fusion toxin and served as the negative control and the other three were reserved for the positive control which was added later. Plates were then incubated at 37°C with 5% CO₂ for 18 hours. Cyclohexamide (Invitrogen) was diluted 1:8 and 10 µL of this dilution was added to each of three wells containing cells only then incubated for the last 15 min. of the 18 hr. incubation serving as the positive control. Cells were pulsed with 1 µCi/well of ³H-Leucine then incubated at 37°C with 5% CO₂ for 1 hour. Cells were harvested onto filter mats (Perkin-Elmer) using a cell harvester (Harvester 96[®] Mach II). The filters were allowed to dry at room temperature overnight then Cpm was measured on a microbeta counter.

2.10. In vivo Functional Analysis

A breeding pair of NOD/SCID IL2 $\gamma^{-/-}$ (*NSG*) mice were purchased from Jackson laboratories and bred in our rodent barrier facilities for use in this study. All animal care procedures and experiments were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care (SRAC). MGH is an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) recognized research institution.

All *NSG* mice were given injections of 10 million porcine CD80⁺ tumor cells (LCL13271) IV via the tail vein. Six mice were injected with 50 µg/kg of porcine CTLA-4 fusion toxin (NonGly version) on day 0 and the drug was administered IP twice a day for 4 days and then once a day every 3 days for 9 days. Controls (n=8) received the tumor cells without the fusion toxin and an additional two mice were given the tumor cells and treated with the drug vehicle (PBS). Injected animals were then observed daily for signs and symptoms of illness and scored biweekly based on several parameters (Schenk et al manuscript in preparation) namely: respiratory effort (0–3), weight loss/gain (0–2), fur integrity (0–3), provoked (0–3) and non-provoked activity (0–1), posture (0–3), abdominal distention (0–3), abdominal palpation (0–3) and body condition score (0–3). The highest score in each category represents the worst possible condition for that parameter. The highest possible score on the scoring system is a 24. Mice were humanely euthanized and necropsy was performed after a score of 12 or higher was achieved or when an animal lost more than 15% of its pre-injection body weight.

3. Results

3.1. Expression and Purification of the Porcine CTLA-4 Fusion Toxins

Porcine CTLA-4 fusion toxins are composed of two domains: 1) the truncated diphtheria toxin DT390 and 2) soluble porcine CTLA-4. Codon-optimized glycosylated or non-*N*-glycosylated soluble porcine CTLA-4 DNA was cloned into the yeast *Pichia pastoris* expression vector pwPICZalpha-A-dmDT390 between *NcoI* and *EcoRI* (Wang et al., 2011). To facilitate the later purification we added a six histidine (6xHis) tag to the C-terminus of the constructs. As shown in Figure 1 we generated three versions of the porcine CTLA-4 fusion toxin: 1) Gly = monovalent glycosylated soluble porcine CTLA-4 fusion toxin (DT390-pCTLA-4-Gly); 2) NonGly = monovalent non-*N*-glycosylated soluble porcine CTLA-4 fusion toxin (DT390-pCTLA-4-Non-*N*-Gly) and 3) Bi-NonGly = bivalent non-*N*-glycosylated soluble porcine CTLA-4 fusion toxin (DT390-bi-pCTLA-4-Non-*N*-Gly). The two non-*N*-glycosylated soluble porcine CTLA-4 proteins which form the Bi-NonGly fusion toxin were joined by a (G₄S)₃ linker containing three tandem chains each made up of four glycine residues and one serine residue. Each of the fusion toxins contains one G₄S linker between the DT390 domain and porcine CTLA-4.

The recombinant porcine CTLA-4 fusion toxins were expressed in yeast *Pichia pastoris* using shake flasks as described in the Materials and Methods section above. The secreted porcine CTLA-4 fusion toxin in the supernatant was captured directly by a Ni-Sepharose fast flow resin. Poros 50HQ, a strong anionic exchange resin was used for the second purification. We obtained pure porcine CTLA-4 fusion toxins following the two-step purification (Figure 2A). Western blot analysis confirmed the porcine CTLA-4 fusion toxins using mouse anti-6xHis monoclonal antibody and mouse anti-diphtheria toxin monoclonal antibody (Figure 2B). The final purification yield was ~10 mg per liter of the original harvested supernatant.

3.2. Binding Analysis of the Porcine CTLA-4 Fusion Toxins to Porcine CD80

Porcine CTLA-4 fusion toxins were biotinylated and the binding affinities to porcine CD80 were analyzed by flow cytometry using LCL13271 cells. As shown in Figure 3A, all three porcine CTLA-4 fusion toxins bound to porcine CD80 in a dose dependent manner. Of the three isoforms, the NonGly was stronger than the Gly and the Bi-NonGly is the strongest isoform. The K_D was determined by plotting MFI *versus* a wide range of concentrations of the biotinylated porcine CTLA-4 fusion toxins (Figure 3B). Fitting of the non-linear least-squares curve revealed a K_D of 24.9 nM for Gly, 12 nM for NonGly and 3.8 nM for Bi-NonGly (see the m3 value of the inset table in Figure 3B1–B3). Following the addition of DT390 the affinity of the Gly isoform for porcine CD80 was less favorable as determined by an increased K_D from 13 nM [our previously published data (Peraino et al., 2012)] to 24.9 nM whereas the binding affinity of the NonGly reagent remained at a K_D of 12 nM. With a K_D of 3.8 nM it can be inferred that the Bi-NonGly fusion toxin has the strongest affinity for porcine CD80.

To confirm the binding specificity of the porcine CTLA-4 fusion toxins to porcine CD80 a dose-dependent blocking assay was performed in which unlabeled fusion toxins were first added to LCL13271 cells thereby impeding the ability of either biotinylated hamster anti-mouse CD80 mAb (clone 16-10A1) or biotinylated soluble porcine CTLA-4 to bind to the cells. These experiments reaffirmed that the porcine CTLA-4 fusion toxins were binding specifically to porcine CD80 on LCL13271 cells (data not shown).

3.3. Protein Synthesis Inhibition Analysis of the Porcine CTLA-4 Fusion Toxins

A protein synthesis inhibition assay using LCL13271 cells was performed to assess the functional ability of these porcine CTLA-4 fusion toxins *in vitro*. As shown in Figure 4, the NonGly and Bi-NonGly isoforms proved to be more efficient in inhibiting protein synthesis in target cells than the Gly isoform. These data demonstrate that the non-*N*-glycosylated porcine CTLA-4 versions have the most optimal functional ability. We speculate that these results may be due to a higher binding affinity of the non-*N*-glycosylated isoforms to the LCL13271 target cells as supported by above K_D analysis. DT390 alone and pCTLA-4-Non-*N*-Gly¹ alone were included as negative controls. pCTLA-4-Non-*N*-Gly alone had completely no ability to inhibit protein synthesis. DT390 alone was only able to inhibit protein synthesis at the highest concentration analyzed (2 μ M) due to non-specific toxicity.

3.4. In vivo Functional Analysis of Porcine CTLA-4 Fusion Toxin Using a Newly Developed Tumor-Bearing NSG Mouse Model

A porcine CD80-expressing tumor (LCL13271)-bearing *NSG* mouse model developed in our lab (Schenk et al., manuscript in preparation) was used to assess the *in vivo* function of the NonGly porcine CTLA-4 fusion toxin. *NSG* mice injected with LCL13271 tumor cells and the NonGly fusion toxin demonstrated prolonged survival in comparison to the untreated mice from a median of 71 days to 88 days ($p < 0.01$) (Figure 5). Mice that received the NonGly fusion toxin alone did not show any evidence of toxicity at the 50 μ g/kg dose. All animals that were injected with LCL13271 cells succumbed to tumors, demonstrated by gross pathology and histopathology (data not shown). Overall, prolonged forbearance was observed in mice that were treated with the porcine CTLA-4 NonGly fusion toxin. Further studies will be needed in order to modulate the tumor burden/fusion toxin dose.

4. Discussion

Human CTLA-4-Ig (abatacept or belatacept), is a United States FDA approved recombinant protein drug capable of binding to human CD80- or CD86-expressing APCs. Our newly developed porcine CTLA-4 fusion toxin carries a double advantage as it is able to both bind to and subsequently deplete the CD80-expressing porcine APCs through protein synthesis inhibition. The ability of these porcine CTLA-4 fusion toxins to bind to porcine CD86 has yet to be assessed.

We have demonstrated that both glycosylated and non-*N*-glycosylated soluble porcine CTLA-4 bind to porcine CD80 on LCL13271 cells with equal affinity ($K_D = 13$ nM) and that each of these constructs is effective in impeding the binding of an anti-CD80 mAb (Peraino et al., 2012). However, when linked to a truncated diphtheria toxin, the non-*N*-glycosylated soluble porcine CTLA-4 fusion toxin binds to the same porcine CD80 on the same LCL13271 cells with notably higher affinity compared to the glycosylated isoform (Figure 3). We speculate that the high-mannose *N*-glycosylation of yeast *Pichia pastoris* in which these toxins were expressed specifically influences the protein conformation thus affecting the binding affinity and may also hinder the internalization of the fusion toxin thereby obstructing the functional ability of the glycosylated isoform. In consequence, removal of the *N*-glycosylation sites appears to be a useful approach to ensure that the *Pichia pastoris* expressed fusion toxin is most optimally functional.

In this study the NonGly fusion toxin proved to be capable of targeting CD80⁺ LCL13271 tumor cells *in vivo* using an *NSG* tumor-bearing mouse model. Despite the statistically significant advantage observed between mice that were treated with the NonGly fusion toxin and the untreated controls, all animals eventually succumbed to the tumors. Further optimization of the fusion toxin treatment (and likely combination with other therapies) may be required. It is also necessary to add the truncated DT390 alone and soluble porcine

CTLA-4 controls in future in vivo studies. The system used, though reliable, is artificial due to the immuno-compromised nature of the mice. Pre-clinical application of the fusion toxin will be required in a swine model to further validate the murine studies.

CD80 was recently recognized as a second binding ligand of programmed death ligand 1 (PDL1). The interaction between CD80 and PDL1 generates a negative signal to down-regulate the immune response (Paterson et al., 2011; Yang et al., 2011), thus eliminating CD80⁺ cells may unfavorably affect PDL1 function. Furthermore, it has also been reported that the B7-H2/ICOS pathway generates a positive regulation signal and B7-H2 was recognized as the third ligand of CTLA-4 (Wekerle et al., 2012). CD80⁺ cell depletion via application of our newly developed porcine CTLA-4 fusion toxins may influence any downstream negative or positive regulation effects of the porcine PDL1/CD80 and/or porcine B7-H2/ICOS pathways. The initial motive of this project was to use the porcine CTLA-4 fusion toxin to deplete porcine APCs thereby down-regulating the immune response. However, the following should be considered as possible unintended effects of treatment with this agent: 1) blocking of the porcine CTLA-4 negative regulation signal; 2) blocking of the negative regulation signal of the porcine PDL1/CD80 pathway; 3) blocking of the positive regulation signal of the porcine B7-H2/ICOS pathway; and/or 4) deleterious effects on Tregs (Wekerle et al., 2012). Therefore, it is necessary to study this reagent further in naïve and disease swine models to evaluate the final net effect on immune responses *in vivo*.

Due to an abundance of available mouse models such as mouse EAE, CD28^{-/-} and/or CTLA-4^{-/-}, a mouse CTLA-4 fusion toxin may help to quickly drive this investigation forward. A human CTLA-4 fusion toxin carries far more clinical relevance and has the potential to be used as a pharmaceutical drug for cancer and autoimmune disease treatment along with tolerance induction following transplantation. Human and mouse CTLA-4 fusion toxins are currently under development.

In summary, this newly developed DT390-based porcine CTLA-4 fusion toxin can be used to 1) directly deplete porcine CD80⁺ cells (and possibly porcine CD86⁺ cells, pending further investigation); 2) indirectly regulate the immune response via depletion of porcine APCs to treat autoimmune diseases or induce tolerance following transplantation in a pre-clinical swine model.

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Highlights

- A porcine CTLA-4 fusion toxin was developed
- The fusion toxin was expressed and purified in *Pichia pastoris*
- *In vitro* function was analyzed using a protein synthesis inhibition assay
- *In vivo* function was assessed using a tumor-bearing *NSG* mouse model

Figure 1.
Schematic Representation of the Three Porcine CTLA-4 Fusion Toxins.

Figure 2A

Figure 2B

Figure 2.

SDS Gel and Western Blot Analysis of the Three Porcine CTLA-4 Fusion Toxins. A) SDS Gel Analysis: DT390-pCTLA-4-Gly (57 kDa, left panel). The main protein band in this panel is ~10 kDa higher than the calculated molecular weight because of the two *N*-linked glycosylations. The weak band is ~5 kDa higher than the calculated molecular weight

because of the partial *N*-linked glycosylation. DT390-pCTLA-4-Non-*N*-Gly (57 kDa, middle panel). DT390-bi-pCTLA-4-Non-*N*-Gly (71 kDa, right panel). The lower weak bands in this panel are break-down products. B) Western Blot Analysis: mouse anti-His monoclonal antibody (left panel) and mouse anti-diphtheria toxin monoclonal antibody (right panel) were used for first staining. Rat anti-mouse IgG-HRP was used for second staining. Gly: DT390-pCTLA-4-Gly; NonGly: DT390-pCTLA-4-Non-*N*-Gly; Bi-NonGly: DT390-bi-pCTLA-4-Non-*N*-Gly.

A

FACS Binding Analysis Using Porcine CD80⁺ LCL 13271 Cells

B3

KD of DT390-bi-pCTLA-4-Non- N-Gly

Figure 3.

A) Flow Cytometry Binding Analysis of Biotinylated DT390-pCTLA-4-Gly (left panel), DT390-pCTLA-4-Non-*N*-Gly (middle panel) and DT390-bi-pCTLA-4-Non-*N*-Gly (right panel) to the Porcine CD80-Expressing B-cell Lymphoma Line LCL 13271. Only second staining control (PE-conjugated streptavidin) and biotinylated hamster anti-mouse CD80 mAb as positive control were also included. The data are representative of multiple individual experiments. B) K_D Determination Using Flow Cytometry and Nonlinear Least Squares Fit. MFI was plotted over a wide range of concentrations of biotinylated DT390-pCTLA-4-Gly (B1), DT390-pCTLA-4-Non-*N*-Gly (B2) and DT390-bi-pCTLA-4-Non-*N*-Gly (B3). The accompanying least-squares fits and parameters are shown based on the hyperbolic equation $y = m1 + m2 * m0 / (m3 + m0)$ where y = MFI at the given biotinylated porcine CTLA-4 fusion toxin concentration, $m0$ = biotinylated porcine CTLA-4 fusion toxin concentration, $m1$ = MFI of zero biotinylated porcine CTLA-4 fusion toxin control, $m2$ = MFI at saturation and $m3 = K_D$. The inset table in B1 shows a fitted K_D of 24.9 nM for DT390-pCTLA-4-Gly. The inset table in B2 shows a fitted K_D of 12 nM for DT390-pCTLA-4-Non-*N*-Gly. The inset table in B3 shows a fitted K_D of 3.8 nM for DT390-pCTLA-4-bi-Non-*N*-Gly.

Figure 4.
In vitro Protein Synthesis Inhibition Analysis of the Three Porcine CTLA-4 Fusion Toxins Using LCL13271 Cells: 1) DT390-pCTLA-4-Gly (red); 2) DT390-pCTLA-4-Non-*N*-Gly (black); and 3) DT390-bi-pCTLA-4-Non-*N*-Gly (pink). DT390 alone (blue) and soluble pCTLA-4-Non-*N*-Gly (green) were included as controls. Y-axis: cpm value by incorporating the tritium-labeled leucine. X-axis: plated porcine CTLA-4 fusion toxin concentration. Cycloheximide (1:8) was used as a positive control. This data is representative of multiple repeated assays.

Figure 5. *In vivo* Functional Analysis of the Porcine CTLA-4 Fusion Toxin
NSG mice were injected with 1.0×10^7 porcine CD80⁺ B-cell lymphoma cells (LCL13271). Mice in the experimental group (n=6) that were treated with DT390-pCTLA-4-Non-*N*-Gly (NonGly) (red curve) had a median survival time of 87 days ($p < 0.01$) compared to 71 days in untreated controls (n=8, no fusion toxin) (blue curve). The schedule of drug administration is pictured in the schematic below the survival curve. The vertical arrows indicate the days on which the DT390-pCTLA-4-Non-*N*-Gly fusion toxin was administered.

Table 1

PCR primers used in this study

DTCTL
5' CCG <u>CTC GAG CCA TGG</u> GGT GGT GGT GGT TCT ATG CAC GTT GCT CAA CCA GCT 3'
XhoI NotI
CTL-E
5' CCG <u>GAA TTC</u> CGC CGC <u>GGA TCC</u> ACC ACC ACC AGA ACC ACC ACC ACC GTC AGA GTC AGG ACA TGG TTC 3'
EcoRI BamHI
CTL-B
5' CCG <u>CTC GAG GGA TCC</u> GGT GGT GGT GGT TCT ATG CAC GTT GCT CAA CCA GCT 3'
XhoI BamHI
CTL161
5' CCG <u>GAA TTC</u> TTA GTG GTG GTG GTG GTG GTC AGA GTC AGG ACA TGG TTC 3'
EcoRI
DT390 For
5' CCG <u>CTC GAG</u> AAG AGA GCT GGT GCT GAC GTC GTC 3'
XhoI
DT390 Rev
5' CCG <u>GAA TTC</u> TTA CCA TGG CAA GAA TGG TTG GGT 3'
EcoRI